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EUROPEAN PATENT APPLICATION

⑪ Application number : **92305769.9**

⑤ Int. Cl.⁵ : **B01D 1/14, C12N 9/96,
C12N 11/02, C07K 17/02**

⑫ Date of filing : **24.06.92**

③ Priority : **26.06.91 GB 9113798**
09.04.92 GB 9207839

④ Date of publication of application :
30.12.92 Bulletin 92/53

⑧ Designated Contracting States :
DE DK FR GB IT NL SE

⑦ Applicant : **PAFRA LIMITED**
Bentalls
Basildon, Essex SS14 3BU (GB)

⑦ Inventor : **Franks, Felix**
7 Wootton Way
Cambridge CB3 9LX (GB)
Inventor : **Hatley, Ross Henry Morris**
114 Limes Road
Hardwick, Cambridge CB3 7XU (GB)
Inventor : **Mathias, Sheila Frances**
Danesmead, Hillside North Pickenham
Swaffham, Norfolk PE37 8JZ (GB)

⑦ Representative : **Ford, Michael Frederick et al**
MEWBURN ELLIS 2 Cursitor Street
London EC4A 1BQ (GB)

⑤ Storage of materials.

⑤ Materials which are not themselves storage-stable at room temperature are made suitable for storage by mixing them with a carrier substance and spray drying the resulting mixture so as to form particles containing both the material and the carrier substance in which the carrier substance is in an amorphous, i.e. glassy or rubbery, state. Formation of such a composition greatly enhances stability. The material stored may be a biological material such as an enzyme, the components of a chemical reaction such as reagents for carrying out an assay, or even viable biological cells.

This invention relates to the stabilisation and storage of materials. The principal envisaged field of application is materials employed in the biochemical field and some pharmaceuticals.

A few biologically active materials (e.g. some proteins) are sufficiently stable that they can be isolated, purified and then stored in solution at room temperature. For most materials however this is not possible and some more elaborate form of stabilisation/storage procedure must be used.

As discussed in our co-pending European application published as EP-A-383569 a number of storage techniques are known but are not universally applicable to materials which give rise to a storage problem.

That pending application discloses the storage of materials by incorporating them into a water-soluble or water-swellaable substance which is in an amorphous, glassy or (much less preferably) rubbery state.

That application discloses the preparation of storable compositions by preparing a solution of the substance(s) to be stored and a water-soluble or swellaable substance, then evaporating water from the solution at room temperature or with some heating. Temperatures of 37°C and 60°C are exemplified. The solutions were simply held in a stationary container during drying.

It is of course considered prudent to minimise the application of heat when drying a material which is not particularly stable. Freeze drying is a prime example of this.

Spray drying is a known process for drying a solution or suspension to a solid, particulate form. The process entails delivering the solution or suspension into a flow of preheated gas, usually air, whereupon water rapidly evaporates from the droplets. It is widely used in the manufacture of detergent powders and in that field it is well known that certain materials are not stable to spray-drying conditions.

Spray drying has been used to kill microbial cells, for instance in dairy products as disclosed by A. Chopin et al, Can J. Microbiol 23, 716 (1977). EP-A-366303 discloses the use of spray drying to dry a cell composition with the intention that the cells will be killed but cell components such as enzymes will be recoverable from the dried composition. Spray drying has been used in attempts to dry microbial cells to a state of suspended animation from which viable cells can be recovered, but even in a relatively favourable case losses of 97% were recorded after 30 days storage at room temperature as disclosed by I.A. Abd el Gawad et al Egyptian Journal of Dairy Science, 17 273 (1989).

Surprisingly, we have now found that spray drying can be used to make storable compositions by drying mixtures of the material(s) to be stored and aqueous solutions of a water-soluble or water-swellaable substance which forms a glassy (or possibly rubbery) state on drying.

According to this invention, therefore, we provide a process of rendering a material suitable for storage comprising spraying into a hot gas stream, an aqueous mixture of the material and a carrier substance which is water-soluble or water-swellaable, thereby drying the mixture to particles in which the said carrier substance is in a glassy or rubbery state, and separating the particles from the gas stream.

This process is of course also a process for preparing a storable composition.

The aqueous mixture of the material for storage and the carrier substance will generally be formed by mixing the material with the carrier substance in the presence of water. However, it is possible that the material to be stored will be provided as a solution which already contains a substance which is able to form a glass and so is suitable as a carrier substance, so that deliberate addition of a carrier substance is unnecessary.

As will be explained in more detail below it is preferred that the composition produced by the drying procedure displays a glass transition temperature of at least 20°C, preferably at least 30°C and possibly well above this e.g. at least 50°C.

The invention may be utilised for stable storage of a single material, or for a mixture of materials which have little or no effect on each other.

However, a further possibility is that the invention is used to produce a composition which contains a plurality of materials which (when in contact with water) form part or all of a reacting system. These materials may be fairly simple chemicals.

Yet another possibility is that the material comprises viable biological cells.

Material Stored (i) inanimate materials

The material(s) stabilised for storage may potentially be any of a wide range of materials which are ordinarily liable to undergo chemical reaction, and so are not stable during storage at ambient temperature of 20°C.

One category of materials to which the invention is applicable is proteins and peptides, including derivatives thereof such as glycoproteins. Such proteins and peptides may be any of: enzymes, transport proteins, e.g. haemoglobin, immunoglobulins, hormones, blood clotting factors, other blood plasma components and pharmacologically active proteins or peptides.

Another category of materials to which the invention is applicable comprises nucleosides, nucleotides, dinucleotides, oligonucleotides (say containing up to four nucleotides) and also enzyme cofactors, whether or

not these are nucleotides. Enzyme substrates in general are materials to which the invention may be applied.

The material for stabilisation and storage may be isolated from a natural source, animal, plant, fungal or bacterial, may be produced by and isolated from cells grown by fermentation in artificial culture, or may be produced by chemical synthesis. Such cells may or may not be genetically transformed cells.

5 The material will need to be soluble in aqueous solution, at least to the extent of forming a dilute solution which can be used for incorporation into the carrier substance.

As mentioned above, a possibility is to store more than one component of a reacting system in a glass. This can be useful for materials which will be required to be used together in, for example, an assay or a diagnostic kit.

10 Storing the materials as a single glassy preparation provides them in a convenient form for eventual use. For instance, if an assay requires a combination of one or more substrates, and/or a cofactor and an enzyme, two or all three could be stored in a glass in the required concentration ratio and be ready for use in the assay.

If multiple materials are stored, they may be mixed together in an aqueous solution and then incorporated together into a glass. Alternatively they may be incorporated individually into separate glasses which are then mixed together.

15 When multiple materials are stored as a single composition (which may be two glasses mixed together) one or more of the materials may be a protein, peptide, nucleoside, nucleotide or enzyme cofactor. It is also possible that the materials may be simpler species. For instance a standard assay procedure may require pyruvate and NADH to be present together. Both can be stored alone with acceptable stability. However, when brought together in aqueous solution they begin to react. If put together in required proportions in the glassy state they do not react and the glass can be stored.

Material to be Stored (ii) cells

25 In a significant development of this invention we have found that the material which is stored may comprise viable biological cells. The composition obtained by spray drying can then contain the cells in a state of suspended animation, and viable cells can be recovered from storage. Cells which may be placed in a storable condition by the method of the invention will preferably be existing as single cells, being either a single cell organism or being cells which are in culture as individual, undifferentiated cells. In particular the cells may be 30 a bacterial culture, which may be isolated from nature or may be a laboratory or industrial bacterial strain including genetically transformed bacteria. The cells may be eukaryotic cells, notably including yeasts but also other fungal cultures. Again the cell culture may be a natural isolate or a laboratory or industrial culture produced by fermentation including genetically transformed strains.

35 The Carrier Substance

A glass is defined as an undercooled liquid with a very high viscosity, that is to say at least 10^{13} Pa.s, probably 10^{14} Pa.s or more.

40 Normally a glass presents the appearance of a homogeneous, transparent, brittle solid which can be ground or milled to a powder. In a glass, diffusive processes take place at extremely low rates, such as microns per year. Chemical or biochemical changes involving more than one reacting moiety are practically inhibited.

Above a temperature known as the glass transition temperature T_g , the viscosity drops rapidly and the glass turns into a rubber, then into a deformable plastic which at even higher temperatures turns into a fluid.

45 The carrier substance employed in this invention must be hydrophilic - either water-soluble or water-swelling - so that water will act as a plasticiser. Many hydrophilic materials, both of a monomeric and a polymeric nature either exist in an amorphous state or can be converted into such an amorphous state which exhibit the glass/rubber transitions characteristic of amorphous macromolecules. They have well defined glass transition temperatures T_g which depend on the molecular weight and on molecular complexity of the substance concerned. T_g is depressed by the addition of diluents. Water is the universal plasticiser for all such hydrophilic 50 materials. Therefore, the glass/rubber transition temperature is adjustable by the addition of water or an aqueous solution.

It will generally be preferred to employ a carrier substance which, on its own, forms a glass rather than a rubber at ambient temperature. Hence it will be preferable that the carrier substance, on its own, is able to exist in a glassy amorphous state with a glass transition temperature T_g above 20°C .

55 Mixtures of substances may be used as carrier substance if the components are miscible as a solid solution. If so, material(s) of lower T_g serve as plasticiser(s) for material(s) of higher T_g .

A composition prepared by a process of this invention will generally have a glass transition temperature T_g below that of the pure carrier substance.

If the dried composition is stored in the glassy state (below T_g) the deterioration of the active material is retarded to the extent that, on practical time-scales, even substances which in solution are extremely labile are found to possess long shelf-lives.

5 Full biochemical activity is maintained, but locked in, throughout this period at temperatures below T_g and can be rapidly released by resolubilization of the glass in an aqueous medium.

If the material to be stored is inanimate, then in order to provide a long storage life, it will generally be desirable that the dried composition has a glass transition temperature of at least 20°C. For achieving this it is desirable that the glass-forming carrier substance, when anhydrous or nearly so, displays a glass transition temperature T_g of at least 40°C, better at least 50°C. There is no theoretical upper limit on T_g . In practice suitable materials have values of T_g below 250°C, usually below 200°C. A desirable range is therefore 50° to 200°C, 10 preferably 60° or 80°C to 150° or 180°C.

If the material to be stored comprises biological cells, the composition containing the cells may well be in an amorphous rubbery state. Thus a composition obtained by the method of the invention and comprising biological cells may have a glass transition temperature T_g below 20°C for instance in the range between 0°C and 20°C even though its carrier substance has a glass transition temperature above 20°C. Such compositions 15 could readily be stored with refrigeration to approximately 0°C so as to be stored in the glassy rather than rubbery state.

If T_g of the final composition made according to the invention is sufficiently high, storage can be at room temperature. However, if T_g of the composition is close to or below room temperature it may be necessary or 20 desirable to refrigerate the composition if storage is for a prolonged period. This is less convenient but still is more economical than freeze-drying.

If a composition is heated above its T_g during storage, it will change to its rubbery state. Even in this condition stored materials are stable for a considerable period of time. Consequently, it may well do no harm if the temperature of a stored composition is allowed to rise above T_g for a limited time, such as during transportation.

25 If a composition is maintained slightly above its T_g (and therefore in a rubbery condition) the storage life will be limited but still considerable and the benefit of the invention will be obtained to a reduced extent.

Conversely, if T_g of the composition is well above room temperature, the composition is better able to withstand storage at an elevated temperature, e.g. in a hot climate.

The carrier substance should be sufficiently chemically inert towards an inanimate material which is to be incorporated in it. An absolute absence of chemical reactivity may not be essential, as long as it is possible to 30 incorporate the material, store the glass, and recover the material without serious degradation through chemical reaction.

If the material to be stored comprises biological cells, the carrier substance should not be toxic to these cells. Indeed the carrier substance may be a nutrient for the cells and able to support cell division so long as 35 the drying operation is carried out at sufficient rapidity that the carrier substance is not consumed by the cells.

As mentioned above, the carrier substance, which very preferably forms a glass, may be deliberately added to form the mixture which is spray dried. For instance it may be added to a solution of the material to be stored.

Many organic substances and mixtures of substances will form a glassy state on cooling from a melt.

40 In this context carbohydrates are an important group of glass forming substances: thus candy is a glassy form of sugar (glucose or sucrose). The T_g for glucose, maltose and maltotriose are respectively 31, 43 and 76°C. (L. Slade and H. Levine, Non-equilibrium behaviour of small carbohydrate-water systems, Pure Appl. Chem. 60 1841 (1988)). Water depresses T_g and for these carbohydrates the depression of T_g by small amounts of moisture is approximately 6°C for each percent of moisture added. We have determined the T_g value for sucrose as 65°C. 45

In addition to straightforward carbohydrates, other polyhydroxy compounds can be used, such as carbohydrate derivatives and chemically modified carbohydrates (i.e. carbohydrates which have undergone chemical reaction to alter substituents on the carbon backbone of the molecule but without alteration of that backbone).

Another important class of glass forming substances are water-soluble or water-swellaable synthetic polymers, such as polyacrylamide. 50

Yet another class of substances which are suitable are proteins and protein hydrolysates. Thus albumin can be used, and so can hydrolysis products of gelatin.

A group of glass forming substances which may in particular be employed are sugar copolymers described in US Patent 3 300 474 and sold by Pharmacia under the Registered Trade Mark "Ficoll". This US patent describes the materials as having molecular weight 5,000 to 1,000,000 and containing sucrose residues linked 55 through ether bridges to bifunctional groups. Such groups may be alkylene of 2, 3 or more carbon atoms but not normally more than 10 carbon atoms. The bifunctional groups serve to connect sugar residues together. These polymers may for example be made by reaction of the sugar with a halohydrin or a bis-epoxy compound.

The suitability of an intended carrier substance, and the amount of material which can be incorporated into it can both be checked by preparing a glassy or rubbery composition with the material incorporated, and then recovering the material without any substantial period of storage.

T_g values can be determined with a differential scanning calorimeter and can be detected as a point at which a plot of heat input against temperature passes through an inflection point - giving a maximum of the first temperature derivative.

As was also mentioned above, a further possibility is that the material which is to be stored may occur in a form which incorporates a suitable carrier substance. It is envisaged in particular that this situation may arise with products derived from blood plasma where the material to be stored is a relatively minor component of the blood plasma and other components which naturally occur in the blood plasma, notably albumin, are able to form a glass on drying. In such a situation there would be no need for separate addition of a glass-forming carrier substance although the possibility is not ruled out.

Processing

The first stage is to provide an aqueous mixture of the material to be stored and the water-soluble or water-swellaable carrier substance. This may be done by mixing the carrier substance, as a powder or as an aqueous solution, with a solution or suspension of the active material to be stored. Alternatively a suitable solution may be available from some other process, without requiring deliberate addition of glass-forming carrier, as mentioned above.

When the invention is applied to the storage of cells, a possibility which has been found suitable for some cells is to suspend the cells in a dilute aqueous solution containing the carrier substance and then subject this to the drying step. To arrive at the suspension, solid carrier material may be dissolved in a suspension of the cells in a dilute aqueous buffer solution. This can lead to a composition with a glass transition temperature above ambient and temperature having very good storage stability.

For some cells, it has been found that survival during drying is better if the cells are dried from a mixture which is rather closer to their normal growth medium. This may for example be carried out by adding carrier substance to an aqueous culture of the cells in their growth medium and drying the resulting mixture. Many bacteriological growth media have a relatively high electrolyte content and this electrolyte or other components is effective to lower the glass transition temperature of the dried product. If this procedure is followed the composition is likely to have a glass transition temperature below ambient temperature making it desirable to store the composition under refrigeration. In this situation the need for refrigerated storage is accepted for the sake of greater survival of cells during the drying operation.

In order to determine whether any cell species can be dried from a simple aqueous suspension or whether it should be dried from something akin to its growth medium, a test can be made by drying some cells by each procedure, then recovering the cells without storing for any substantial period of time and determining the quantity of cells which have survived.

After arriving at a mixture containing the material to be stored and a carrier substance the next step is a spray drying operation in which the above aqueous mixture is sprayed into a hot gas stream. The gas will generally be air but could be some other gas such as nitrogen.

Apparatus to carry out spray drying on a fairly small scale is available from various manufacturers. One is Drytec Ltd, Tonbridge, Kent who manufacture a pilot plant scale dryer. Another manufacturer is Lab-Plant Ltd of Longwood, Huddersfield, England who manufacture a laboratory scale dryer.

Process plant to carry out spray drying on a larger scale is also well known.

The sole drawing is a diagrammatic illustration of laboratory scale spray-drying apparatus.

In this apparatus air from the atmosphere is drawn in by a blower 10 and passes over an electric heater 12 after which the air passes down a main chamber 16. The aqueous mixture to be sprayed is drawn up from a supply vessel 18 by means of a peristaltic metering pump 20 and delivered to a spray nozzle 22 which discharges the aqueous mixture as a fine spray into the stream of hot air coming from the heater 12.

The droplets of spray are dried to solid powder form as they pass down within the main chamber 16. The powder is entrained in the air which has passed down the main chamber 16. This leaves by an exit tube 26 at one side delivering to a cyclone separator 28 which serves to remove entrained solid particles from the air stream. The solid particles which are separated from the air stream in this way are collected as the product in a detachable vessel 30 while the air passes out to atmosphere through an exhaust tube 32. Solids which stick to the wall of the main chamber fall into waste container 24.

A significant parameter in the operation of any spray drying apparatus is the temperature of the gas stream which is admitted to the main chamber and into which the spray is delivered. For the present invention this inlet temperature of the gas stream will generally exceed 80°C, will usually exceed 90°C and may well lie in a range

from 100 to 250/300°C.

The aqueous mixture which is delivered into the gas stream may typically contain from 10 up to 50 or even 250 grams per litre of the carrier substance. The content of material to be stored can vary widely but will often lie within a broad range from 10⁻³% to 10% by weight of the carrier substance. Much lower levels are possible.

5 A substance whose desired activity is present when very dilute might be stored at a concentration as low as 10⁻⁶% by weight of the dried composition. By contrast storage at a concentration of stored material of up to 50% by weight or even more can also be envisaged.

In many instances the carrier substance will form at least 20%, better at least 25% or 30% by weight of the particulate composition formed by drying and usually at least 50% by weight of that particulate composition.

10 The particulate solid compositions produced by discharging an aqueous mixture into a heated gas stream in accordance with the invention will frequently be sufficiently dry for storage without further processing. However it is within the scope of the invention to subject these compositions to further drying such as by subjecting them to sub-atmospheric pressure possibly accompanied by moderate heating so as to reduce any residual moisture content.

15 When the material to be stored comprises biological cells the moisture content of a composition produced by a method of the invention would typically be in the range 3 to 9% by weight. A low moisture content enhances stability.

The suitability of conditions for preparing a storable composition can be checked by carrying out the preparation but recovering the material without any substantial period of storage and determining the proportion which has survived. As mentioned above storage stability can if desired be tested by storage at a temperature above ambient, although not above the T_g value of the glass.

Recovery from storage

25 Recovery of stored material from a composition produced by drying in accordance with this invention can be effected by simply adding water or aqueous solution to a quantity of the composition with the active material therein. If the carrier substance is water-soluble the result is a solution of the material and the carrier substance.

Separation by chromatography to isolate a stored, active, inanimate material from the carrier substance is possible. However, in general it will be neither desirable nor necessary. Instead the carrier substance is chosen so that it will not interfere with the use (e.g. an assay) of the stored, active material.

30 In the case of a water-swellable carrier substance, it will remain out of solution, perhaps as a gel, and the solution of the material can be separated by centrifugation if required.

A further aspect of this invention is use of a composition prepared according to the invention to provide a solution of the stored material, by addition of water or aqueous solution to the composition. The application to which the solution of recovered material is put may or may not be a therapeutic application.

Example 1

40 The active material to be placed in a storable form was lactate dehydrogenase (LDH) type XI (ex rabbit muscle) from Sigma Chemical Co. The glass forming carrier substance employed was Ficoll 400 DL (Pharmacia, Reg. Trade Mark) which is a copolymer of sucrose and epichlorohydrin. It is water-soluble and has a T_g of 97°C.

8 g of Ficoll was added to 200 ml of 0.01 M phosphate buffer pH 7 and stirred at ambient temperature until a clear solution was obtained. This solution was then cooled to, and stored at, 4°C until use. All solutions were used within 72 hr. To 200 ml of the phosphate buffer/Ficoll solution (at 4°C) 10 mg of LDH was added. The resulting solution was then passed through a spray-drier (Drytec, pilot scale drier) to give a dry powder containing 1.25 mg LDH/g powder. An air inlet temperature of 210°C (at the air inlet to the drying chamber) was employed; this produced an air temperature of 70°C at the entry to the cyclone separator.

50 The dried material was a particulate solid. This was divided into 2 g portions and placed into vials. The vials were sealed under a normal atmosphere and stored at ambient temperature (fluctuating between 10 and 35°C). Portions of powder were periodically removed and the vials resealed. Assays were performed following drying, and periodically thereafter. An assay of the solution prior to drying was used as the control.

The actual enzyme activity was determined by the following procedure (Hatley, Franks and Mathias, Process Biochemistry, December 1987 page 170) and based on a minimum of nine replicates. The powder was dissolved in phosphate buffer to give a test solution calculated to contain 1 µg protein per ml (for the control sample a portion of the solution to be dried was taken and diluted to 1 µg per ml).

55 Activity of the test solution was then measured: 2.7 ml of 0.01 phosphate buffer pH 7, 0.1 ml of 2mg/ml NADH and 0.1 ml of 10 mM pyruvate were placed into a cuvette of light path 10 mm. The cuvette was capped

and shaken. 0.1 ml of the test solution was added and the cuvette again capped and shaken. The absorbance at 340 nm was recorded at 30 s intervals for a total of three minutes. The temperature of the solution was also noted. The absorbance change per minute, δA , was calculated. The enzyme activity was then calculated as follows:

$$\text{LDH activity (units/mg)} = \frac{\delta A \times \text{TCF}}{6.25 \times C}$$

where:

δA = the absorbance change per minute

6.25 = a correction factor for the molar absorbance of NADH.

10 TCF = a temperature correction factor applied to all assays performed at temperatures other than 25°C

C = the concentration of protein in mg/ml

The control (unprocessed) solution had an activity of 322 U/mg protein. This was taken as 100% and all subsequent assays quoted relative to this value. Enzyme activities were:

15	Before Drying	<u>Storage period after drying (days)</u>				
		0	12	33	91	138
20	100%	82%	83%	86%	71%	100%

Product T_g was determined throughout the storage period. The value decreased from 79°C to 64°C as moisture entered the product as it was repeatedly opened and resealed. However, throughout the experiment the product remained in the form of a glass at the storage temperature.

The results show that enzyme activity was effectively preserved intact through the spray drying procedure and subsequent storage.

Example 2

30 Example 1 was repeated with an air inlet temperature of 130°C. This produced an air temperature of 60°C at the entry to the cyclone separator. After 138 days storage, enzyme activity was 112% of the control value.

Example 3

35 Example 1 was repeated with two variations. 50 mg of LDH was added to 200 ml of the phosphate buffer/Ficoll solution (at 4°C). Spray drying gave a dry powder containing 6.25 mg LDH/g powder. Air inlet temperature was 150°C. This produced an air temperature of 70°C at the entry to the cyclone separator.

40 After 138 days storage enzyme activity was 117% of the control value.

Example 4

45 The active material was alcohol oxidase from Provesta Enzymes. The glass forming substance employed was Ficoll 400 DL (Pharmacia, Reg. Trade Mark) as used in Example 1.

8 g of Ficoll was added to 200 ml of 0.1 M phosphate buffer pH 7.5 and stirred at ambient temperature until a clear solution was obtained. This solution was then cooled to, and stored at, 4°C until use. All solutions were used within 72 hr. 100 µg (100 units) of alcohol oxidase was added to 200 ml of the phosphate buffer/Ficoll solution (at 4°C). The resulting solution was then passed through a spray-drier (Drytec pilot scale drier) to give 50 a dry powder calculated to contain 0.0125 U alcohol oxidase/g powder. An air inlet temperature of 150°C was employed; this produced an air temperature of 70°C at the entry to the cyclone separator.

The dried material was divided into 2 g portions and placed into vials. The vials were sealed under a normal atmosphere and stored at ambient temperature (fluctuating between 10 and 35°C). Portions of powder were periodically removed and the vials resealed. Assays were performed prior to drying and following drying.

55 As a comparison freeze-dried samples were prepared using principles described in the literature, (F Franks. Cryo-Letters 11, 93-110). 300 mg of Ficoll was dissolved in 20 ml of 0.1 M phosphate buffer pH 7. 1000 units of alcohol oxidase was added to the solution. The solution was divided into 0.2 ml portions in ten 5 ml vials. These were frozen to -30°C in a small laboratory freeze-drier. A vacuum of 1×10^{-1} mbar was applied and the

samples dried for 24 hr. The vacuum was reduced to its minimum setting of 5×10^{-2} mbar and the temperature raised at 5°C/hr to 30°C . After holding the sample at this temperature for two hours the vials were removed and sealed with Bakelite screw-caps. Each vial was calculated to contain 1 unit of enzyme, assuming no activity had been lost during processing. Enzyme activity was assayed before and after freeze drying.

As a further comparison, samples of solution similar to those which were freeze dried, were dried in stationary vials, as described in our published European application EP-A-383569. Enzyme activity was assayed before and after drying.

Assays of enzyme activity were performed as follows: Dried powder was dissolved in phosphate buffer to give a test solution calculated to be 0.1 U per ml (for the control sample a portion of the solution to be dried was taken and serially diluted to 0.1 U per ml).

A stock solution was prepared containing:

16 mg 2,2'-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)

2 ml absolute ethanol

1 ml of 1 mg/ml horseradish peroxidase in distilled water All made up to 100 ml with 0.1 M potassium phosphate buffer, pH 7.5.

Another stock solution was:

0.1 M potassium phosphate buffer, pH 7.5.

2.5 ml of the ABTS stock solution was pipetted into a 3 ml cuvette, 25 μl of the enzyme solution with an expected activity of 0.1 U alcohol oxidase per ml was added. The cuvette was closed and its contents mixed by inversion. The cuvette was placed in a spectrophotometer and the absorbance change over 3 min at 390 nm recorded.

The absorbance change per minute was determined and multiplied by 3.06 (the extinction coefficient plus the cuvette dilution factor) to give the concentration of enzyme in the solution. Activity retention was calculated by dividing the measured value by 0.1 (the expected activity in the solution) and multiplying by 100 to give a percentage.

It was found that after drying the activity as a percentage of that before drying was:

Freeze drying	35%
Drying in stationary vial	38%
Spray drying	52%

Suppliers' catalogues show that commercial freeze drying of alcohol oxidase reduces activity to 25%.

The spray dried and freeze dried alcohol oxidase were stored at 35°C and activity was assessed at intervals. The freeze dried material lost all activity in 20 days. After 30 days the spray dried material retained 90% of its activity assayed directly after spray drying.

Example 5

Example 1 was repeated, replacing Ficoll with Byco A which is a cold water soluble protein obtained from gelatin by enzymic hydrolysis. It has a T_g value of 126°C . Byco A is available from Croda Colloids Ltd, Widnes, Cheshire, England.

As in Example 1 enzyme activity was measured both directly following drying and after a period of storage. The measured values of enzyme activity were 88% directly following drying and 113% after 103 days storage at ambient temperature.

Example 6

As in Example 1, the active material was lactate dehydrogenase (LDH) type XI and the glass forming substance was Ficoll 400 DL.

10 g of Ficoll was added to 100 ml of 0.01 M phosphate buffer pH 7 and stirred at ambient temperature until a clear solution was obtained. This solution was then cooled to, and stored at, 4°C until use. All solutions were used within 72 hr. 10 mg of LDH was added to 200 ml of the phosphate buffer/Ficoll solution (at 4°C). The solution was then passed through a spray-drier (Lab-Plant SD-04) to give a dry powder containing 1 mg LDH/g powder. An air inlet temperature of 170°C was employed; this produced an air outlet temperature of 75°C . The dried material was sealed in a collection bottle in a normal atmosphere. The samples were stored at ambient temperature (fluctuating between 10 and 35°C). Portions of powder were periodically removed and assays performed as for Example 1.

Enzyme activities were:

<u>Storage period (days)</u>			
	Before Drying	0	61
5	100%	91%	103%

Example 7

10 This example describes converting biological cells into a storable composition by a method embodying the invention and then recovering the cells after varying periods of time.

The amount of aqueous solution used in recovery of cells was chosen such that, if there was survival of every cell, the concentration of cells in the recovered suspension would be the same as in the initial suspension before drying. The concentrations of viable cells in these suspensions, before drying and after recovery, were
15 assayed by using a standard quantity of the suspension to make an agar plate. The plate is then incubated and the number of growing colonies was counted.

5 g Ficoll 400 DL (Pharmacia, Reg Trade Mark) was dissolved in 100 ml growth medium. 5 ml Lactobacillus bulgaricus cells in growth medium were added to the Ficoll solution to give a cell suspension of approximately 1×10^7 cells/ml. The suspension was then spray dried, using a laboratory scale spray drier, (Lab-Plant SD-04), with input temperature 190°C and output temperature 104°C, and collected as a dry powder. The powder
20 was immediately dispensed into several small vials and the vials were capped and stored at 4°C.

Viability testing:

25 Before drying (control):

0.5 ml cell suspension was transferred to a sterile tube containing 9.5 ml sterile growth medium. Serial dilutions were made in the manner of Miles and Misra. 1 ml cell suspension from an appropriate dilution was transferred to a 9 cm sterile petri dish and mixed with 10 ml molten growth medium at approximately 37°C. The agar plate was then allowed to set and incubated for 48 h at 35°C. The colony forming units were then counted.

30 After drying:

0.5 ml growth medium was added to 0.049 g dried powder (calculated weight of solids in 0.5 ml original cell suspension) and left to stand at room temperature for 30 min to ensure full rehydration. The assay was continued as described above and the number of colony forming units was compared to the number found in the sample assayed before drying.

35 Further dried samples were assayed at intervals.

Results:

40	Storage time	Cfu's in 10^{-5} dilution	% of control
	Before drying (control)	1365	
45	Directly after drying	344	25
	2 weeks	345	25
50	4 weeks	319	23

Example 8

55 5 g Dextrin 10 (maltodextrin, ex Fluka) was dissolved in 100 ml 10% skimmed milk solution. 5 ml Lactobacillus bulgaricus cells in growth medium were then added to the solution to give a cell suspension of approximately 1×10^7 cells/ml. The suspension was then spray dried, using a laboratory scale spray drier, (Lab-Plant SD-04), with input temperature 160°C and output temperature 89°C, and collected as a dry powder. The powder

was immediately dispensed into several small vials and the vials were capped and stored at 4°C. Viability testing was carried out as in the previous Example. For testing after drying 0.5ml growth medium was added to 0.076g dried powder as the calculated weight of solids in 0.5ml of the original cell suspension.

5 Results:

	Storage time	Cfu's in 10^{-5} dilution	% of control
10	Before drying (control)	1321	
	Directly after drying	543	41
15	2 weeks	551	42
	4 weeks	392	30

20 The moisture content of the spray dried powder was measured by a Karl Fischer coulometric method and found to be 5.2 %.

Example 9

25 5 g raffinose was dissolved in 100 ml growth medium. 5 ml Lactobacillus bulgaricus cells in growth medium were added to the solution to give a cell suspension of approximately 1×10^7 cells/ml. The suspension was then spray dried, using a laboratory scale spray drier, (Lab-Plant SD-04), with input temperature 190°C and output temperature 108°C, and collected as a dry powder. The powder was immediately dispensed into several
30 small vials and the vials were capped and stored at 4°C. Viability testing was carried out as in Example 7.

Results:

	Storage time	Cfu's in 10^{-5} dilution	% of control
35	Before drying (control)	1620	
40	Directly after drying	127	8
45	2 weeks	174	11
	4 weeks	176	11
	8 weeks	117	7

50 Example 10

55 5 g sodium glutamate was dissolved in 100 ml growth medium. 5 ml Lactobacillus bulgaricus cells in growth medium were added to the solution to give a cell suspension of approximately 1×10^7 cells/ml. The suspension was then spray dried, using a laboratory scale spray drier, (Lab-Plant SD-04), with input temperature 190°C and output temperature 114°C, and collected as a dry powder. The powder was immediately dispensed into several small vials and the vials were capped and stored at 4°C. Viability testing was carried out as in Example 7.

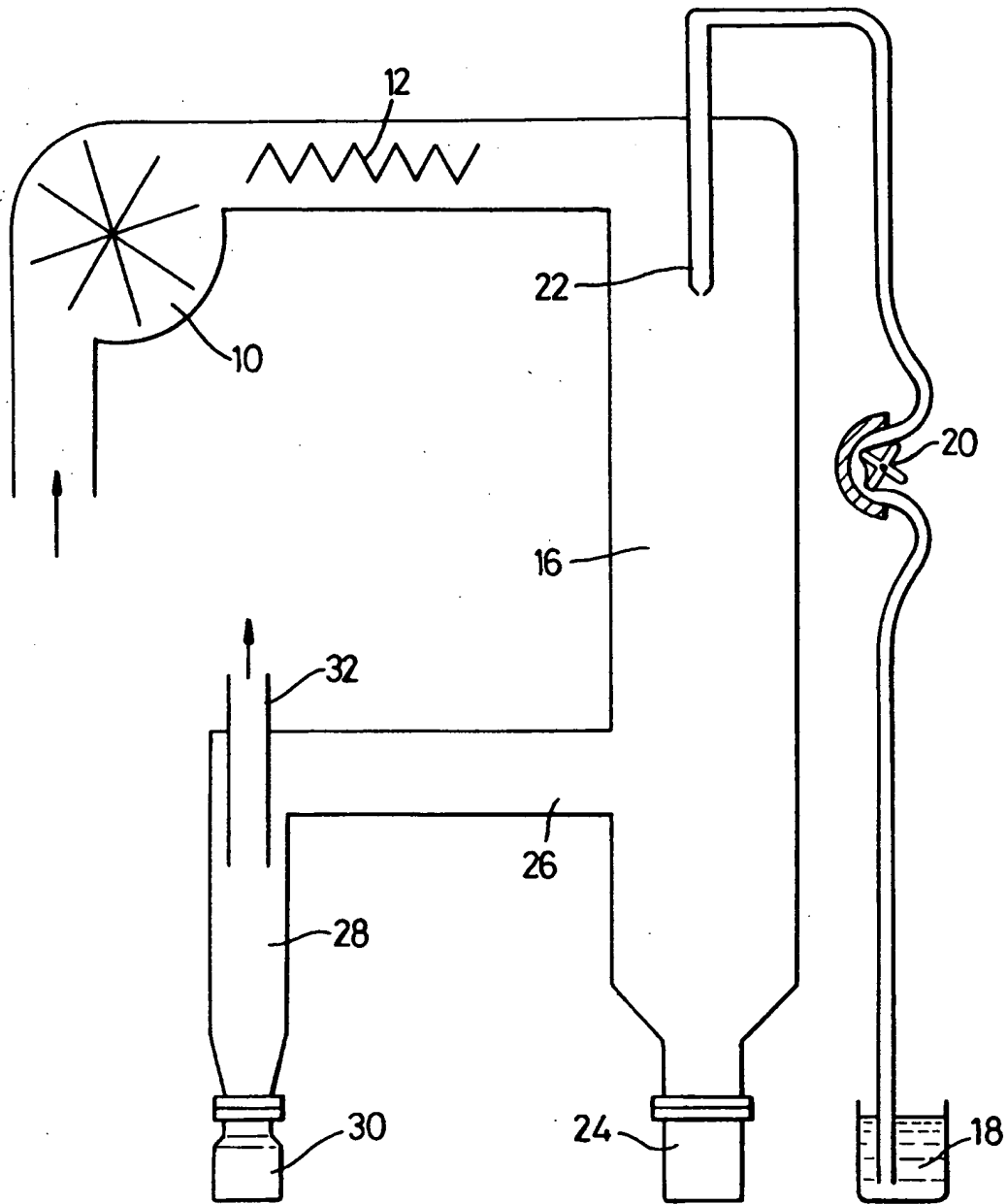
Results:

	Storage time	Cfu's in 10^{-5} dilution	% of control
5	Before drying (control)	1472	
10	Directly after	515	35
	2 weeks	516	35
	4 weeks	534	36
15	8 weeks	451	31

20 **Claims**

1. A process of rendering a material suitable for storage comprising spraying into a hot gas stream an aqueous mixture of the said material and a carrier substance which is water-soluble or water-swella-
25 ble, thereby drying the mixture to particles which contain the material and the carrier substance and in which the said carrier substance is in a glassy or rubbery state, and separating the particles from the gas stream.
2. A process according to claim 1 wherein the carrier substance, when on its own, is able to exist in a glassy amorphous state with a glass transition temperature above 20°C.
- 30 3. A process according to claim 1 or claim 2 wherein the composition produced by the drying procedure displays a glass transition temperature of at least 20°C.
4. A process according to claim 1 or claim 2 wherein the composition produced by the drying procedure displays a glass transition temperature of at least 50°C.
- 35 5. A process according to any one of claims 1 to 4 wherein the carrier substance forms at least 20% by weight of the particles formed by drying.
6. A process according to any one of the preceding claims wherein the material which is rendered suitable for storage is selected from proteins, peptides, nucleosides, nucleotides, dinucleotides, oligonucleotides
40 and enzyme cofactors.
7. A process according to any one of claims 1 to 5 wherein the material to be stored comprises viable biological cells.
- 45 8. A process according to claim 8 wherein the aqueous mixture sprayed into the gas stream comprises a suspension of the cells in growth medium for the cells.
9. A process according to any one of the preceding claims wherein the carrier substance is a polyhydroxy compound, preferably carbohydrate, protein, a protein hydrolysis product, or a sugar polymer containing
50 sugar residues linked through ether bridges to bifunctional groups other than carbohydrate.
10. A storable composition produced by the process of any one of the preceding claims.

55





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 92305769.9
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D, A	EP - A - 0 383 569 (PAFRA LIMITED) * Claims *	1-3, 6, 9, 10	B 01 D 1/14 C 12 N 9/96 C 12 N 11/02 C 07 K 17/02
A	US - A - 4 830 858 (PAYNE) * Claims *	1, 5, 6, 9, 10	
A	US - A - 3 666 496 (HONEY) * Claims *	1, 5	
A	DE - A - 2 415 159 (HOECHST AG) * Totality *	1	
A	DE - A - 1 812 574 (RIEDEL-DE HAEN AG) * Claims *	1	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			B 01 D C 12 N A 23 B A 23 C A 61 J A 61 K C 11 D C 07 K F 26 B
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 31-08-1992	Examiner BECKER
CATEGORY OF CITED DOCUMENTS		I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			

EPO FORM 1503 03.81 (P0401)

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11) Publication number:

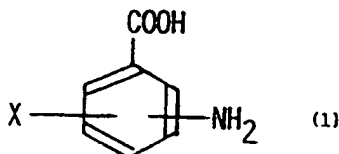
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(12)

EUROPEAN PATENT APPLICATION
published in accordance with Art.
158(3) EPC

(21) Application number: **91903819.0**(51) Int. Cl.⁵: **A61K 31/195**(22) Date of filing: **08.02.91**(86) International application number:
PCT/JP91/00200(87) International publication number:
WO 91/11997 (22.08.91 91/19)(30) Priority: **19.02.90 JP 39241/90**(43) Date of publication of application:
18.03.92 Bulletin 92/12(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE(71) Applicant: **Senju Pharmaceutical Co., Ltd.**
5-8, Hiranomachi 2-chome, Chuo-Ku
Osaka-shi, Osaka 541(JP)(72) Inventor: **INOUE, Jun**
13-57, Shojaku 4-chome, Settsu-shi
Osaka 566(JP)(74) Representative: **Kinzebach, Werner, Dr. et al**
Patentanwälte Reitstötter, Kinzebach und
Partner Sternwartstrasse 4 Postfach 86 06 49
W-8000 München 86(DE)(54) **MAILLARD REACTION INHIBITOR.**

(57) A Maillard reaction inhibitor containing a substance represented by general formula (I), a pharmaceutically acceptable ester thereof, and a pharmaceutically acceptable salt of the substance or the ester, wherein X represents hydroxyl or nitro. It is used for treating or preventing various complications of diabetes, such as coronary artery disease, peripheral circulatory disturbance, cerebrovascular disease, neurosis, nephropathy, arteriosclerosis, arthrosclerosis, cataract and retinitis, and similar diseases caused by aging, such as atherosclerosis, coronary heart disease, cerebrovascular disease, senile cataract, and so forth.



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Technical Field

This invention relates to the inhibition of denaturation reaction of proteins by reductive sugars such as glucose, which is known by the name of Maillard's reaction. More specifically this invention relates to the inhibition of the formation of Amadori rearrangement products which originate from non-enzymatic bond formation between glucose and proteins.

Background Art

The reaction in which proteins turn brown by reacting non-enzymatically with reductive sugars such as glucose (hereinafter referred to as "the glycosylation") was first reported by Maillard in 1912 [Maillard, L.C., Compt. Rend. Soc. Biol., 72:599 (1912).] Since then, the reaction has been widely recognized by the name of Maillard's reaction in the field of food chemistry. For example, it has been noted that proteins react with glucose in stored or heated food, generate a brown color and finally are denaturated by formation of cross-linkings among molecules.

Later, attention was directed to reactions of glucose with proteins which may occur in living bodies when Rahbar reported that the level of Hb_{A1c}, a minor component of hemoglobin, was found elevated in red blood cells of diabetic patients [Rahbar, S., Clin. Chim. Acta, 22:296 (1968).] And, through structural analysis of Hb_{A1c}, it has been confirmed that Maillard's reaction occurs in living bodies.

The mechanism of Maillard's reaction in living bodies has been presented by Brownlee et al. [Brownlee, M. et al., Science, 232:1629 (1986).] The reaction proceeds as follows.

At first, the aldehyde group of the open-ring structure of glucose reacts with an amino group in protein molecule to form a schiff's base. The resulting schiff's base is unstable and is rapidly converted non-enzymatically into Amadori rearrangement product via intra-molecular rearrangement reaction. If this protein is maintained for a long period of time within the body, the rearranged product undergoes a gradual dehydration reaction to form a new glucose derivative. This derivative then irreversibly forms cross-linkings with a variety of molecules including proteins to form bridges among molecules, thus yielding aggregation products of, chiefly, proteins.

This type of product resulting from advanced reactions of glycosylated proteins is usually abbreviated to AGE (Advanced Glycosylation End product.)

In parallel to the formation of AGE, biological adaptability of the protein is lowered, and the protein becomes less soluble and more resistant to proteases and, in many cases, turns yellow-brown and becomes fluorescent.

Though also observed in healthy human, Maillard's reaction is markedly noted in those with diabetes mellitus, which is characterized by the elevation of blood glucose. Maillard's reaction is especially notable in proteins with a slower rate of metabolic turnover, for example crystallins, which are the structural proteins in the lens, and collagens. While a variety of disorders, for example neuropathy, cataract, nephropathy, retinopathy, arthrosclerosis and atherosclerosis, are noted as complications of diabetes mellitus, these disorders bear a very close resemblance with disorders noted quite frequently in the aged human.

It, therefore, is regarded that AGE is also formed gradually from proteins with a slower turnover rate by glycosylation with glucose even at a normal level of blood sugar.

With this background, efforts have been made to find compounds which may inhibit Maillard's reaction within living bodies. An example of such efforts has been shown by Brownlee as cited who reported that aminoguanidine inhibits Maillard's reaction in vitro and suppresses AGE formation in arterial walls of diabetic rats in vivo. In Japanese Patent Publication Kokai No. 142114/87, it has been suggested that aminoguanidine, α -hydrazinohistidine and lysine may block the active carbonyl group of Amadori rearrangement products to inhibit AGE formation. It has also been disclosed that different compounds may suppress Maillard's reaction. Such compounds include thiosemicarbazides, 1,3-diaminoguanidine and benzoylhydrazine (Japanese Patent Publication Kokai No. 56614/89), and various derivatives of guanidine (Japanese Patent Publication Kokai No. 83059/89.)

In the patent publications cited above, researches for inhibitors of Maillard's reaction were made using the amount of AGE, the end product of Maillard's reaction, as an index. The present inventor, instead, took the inhibition of formation of Amadori rearrangement product as an index in the investigation. This was based on an estimation that a markedly effective inhibition of Maillard's reaction may be expected by inhibiting the very formation of Amadori rearrangement product, which is the immediate causing factor in protein aggregation process in Maillard's reaction.

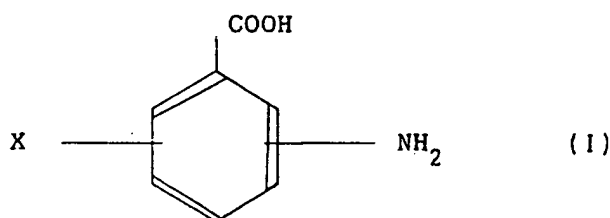
Bruggemann et al. [J. Bruggemann et al., Lebensm. Unters. Forsch., 137:137-143 (1968)] and Finot et al. [P.A. Finot et al., Experientia, 24:1097-1099 (1968)] have reported that the amount of ϵ -N-(furoyl-methyl)-

L-lysine (hereinafter referred to as "furosine"), which is an Amadori rearrangement product resulted from non-enzymatic glycosylation of ϵ -amino residue of lysine in proteins, may be taken as an index of the non-enzymatic glycosylation of protein molecules. The present inventor made an intensive research for the optimal experimental condition for formation of furosine from protein dissolved in water containing glucose, and, according to the condition thus established, evaluated various compounds for the presence and strength of inhibitory effect on furosine formation.

As a result, the present inventor discovered that some of the derivatives of aminobenzoic acids have a potent inhibitory effect on furosine formation. Then, evaluation was continued, which lead to the accomplishment of the present invention.

Disclosure of Invention

Thus the present invention is a pharmaceutical composition for inhibition of Maillard's reaction characterized in that it contains a compound of the formula (I),



wherein X denotes a hydroxyl group or a nitro group, a pharmaceutically acceptable ester thereof or a pharmaceutically acceptable salt of the said compound or the said ester.

Examples of the pharmaceutically acceptable esters of the compound (I) include lower alkyl esters of the carboxyl group of the compound such as methyl ester, ethyl ester, n-propyl ester and isopropyl ester, and esters of the phenolic hydroxyl group of the compound such as esters with lower carboxylic acids including acetic acid ester, oxalic acid ester, malonic acid ester, maleic acid ester and succinic acid ester, and esters with inorganic acids including phosphoric acid ester.

Examples of suitable salts of the compound (I) or pharmaceutically acceptable esters thereof include, in particular, alkali metal salts thereof such as sodium salt and potassium salt, alkaline earth metal salts thereof such as calcium salt and magnesium salt, and salts thereof with inorganic acids such as hydrochloric acid, sulfuric acid and phosphoric acid, or with organic acids such as acetic acid and maleic acid.

The scope of the present invention, however, is not limited by these examples, and salts which are usually accepted as pharmaceuticals are included in the scope of the present invention.

The Maillard's reaction inhibitors of the present invention may be used for the treatment or prophylaxis of a variety of disorders mentioned later which may develop via Maillard's reaction. For the purpose, the inhibitors of Maillard's reaction of the present invention may be administered orally or non-orally. For non-oral administration, the inhibitors may be administered parenterally for systemic purpose or topically, for example, in the form of eye drops.

The Maillard's reaction inhibitor of the present invention may be administered orally at a dose - as the compound (I) - of, generally, 1 to 1,000 mg/day, more preferably 5 to 200 mg/day. For injection, the dose may be generally 0.1 to 100 mg/day, more preferably 1 to 50 mg/day.

For eye drops, it may be applied in the form of liquid at a concentration of, generally, 0.05 to 5.0 w/v %, more preferably 0.1 to 2.0 w/v %.

However, the examples above are not intended to limit the dose range. A suitable dose may be set according to the type and severity of disorders and schedules of treatment in each case.

The Maillard's reaction inhibitor of the present invention may be formed into, for example, tablets, pills, powder, granules or capsules for oral administration, aqueous or non-aqueous solution, suspension or emulsion for injection, or eye drops or eye ointment for ophthalmic topical use.

For preparing pharmaceutical composition of the present invention into the form of tablets for oral administration, ingredients usually incorporated in tablet preparation may suitably be utilized.

Such ingredients include, for example, diluent bases such as hydroxypropylcellulose, crystalline cellulose, corn starch, polyvinylpyrrolidone and magnesium metasilicate aluminate, lubricants such as

magnesium stearate, disintegrators such as fibrinous calcium gluconate, and solubilizers such as glutamic acid and aspartic acid.

For preparing a pharmaceutical composition of the present invention in the form of aqueous injection, ingredients usually incorporated in injectable preparations may suitably be utilized. Such ingredients include, for example, buffering agents such as phosphates, preservatives such as chlorobutanol, stabilizers such as sodium sulfite, and isotonicizers such as sodium chloride.

For preparing a pharmaceutical composition of the present invention into the form of eye drops, ingredients usually incorporated in the formation of eye drops may suitably be utilized. Such ingredients include, for example, buffering agents such as phosphates, borates, acetates and citrates, preservatives such as chlorobutanol, methylparaben, propylparaben, benzalkonium chloride and chlorhexidine digluconate, stabilizers such as sodium sulfite, sodium bisulfite and sodium edetate, isotonicizers such as sodium chloride, potassium chloride, mannitol, sorbitol and glycerol, and solubilizers such as polysorbate 80 and cyclodextrins.

15 (Pharmacological test)

The effect of the Maillard's reaction inhibitors of the present invention was determined as follows using the test compounds listed below.

They are known compounds and were purchased from the market.

- 20 AB-1: 5-hydroxyanthranilic acid
- AB-2: 3-hydroxyanthranilic acid
- AB-3: 4-nitroanthranilic acid
- AB-4: 5-aminosalicylic acid
- AB-5: 4-aminosalicylic acid
- 25 AB-6: 3-aminosalicylic acid
- AB-7: 3-amino-4-hydroxybenzoic acid

(Test methods)

- 30 Sample solutions as shown below were aseptically prepared from bovine serum albumine (No. A-8022, Sigma)(hereinafter referred to as BSA), 50 mM phosphate buffer solution (pH 7.3) and the test compounds listed in Table 1 and aminoguanidine.

- The sample solutions were kept for 4 weeks at 37 °C, and the amount of furosine which was formed by non-enzymatic glycosylation was determined by HPLC according to the method of Schleicher et al. [J. Clin. Biochem., 19:81-87 (1981).] Thus, the sample solutions after reaction were dialyzed, and aliquots of 1 ml were lyophilized and then hydrolyzed by the addition of 1 ml of 6 N hydrochloric acid followed by heating at 100 °C for 20 hours. After removal of hydrochloric acid by evaporation, 1 ml of water was added to each sample, and the samples were subjected to filtration using a filter with the pore size of 0.45 µm. The filtrate was used as the sample for HPLC. ODS-120T (Tosoh Corporation) was used for the column and 7 mM phosphoric acid solution was used as the eluant. The absorbance peak whose ratio of peak area at 280 mm/254 mm was 3.9/1 was regarded as the peak corresponding to furosine.

[Constituents in the phosphate buffer solution]

- 45 Normal sample; 20 mg/ml BSA
- Control sample; 20 mg/ml BSA and 50 mM glucose
- Test sample; 20 mg/ml BSA, 50 mM glucose and 5 mM test compound

Upon the area of the peak of furosine of each sample, the inhibition rate of furosine formation by the test compound was calculated as follows.

- 50 Inhibition rate (%) = $(c-d) \div (c-n) \times 100$

- c; peak area of furosine of the control sample
- 55 d; peak area of furosine of the test sample
- n; peak area of furosine of the normal sample

(Results)

As shown in Table 1, each of the test compounds, AB-1 to AB-7, exhibited a remarkably potent inhibitory effect in comparison with aminoguanidine, a known inhibitor of Maillard's reaction.

Table 1

Test compound	Inhibition rate (%)
AB-1	94.1
AB-2	69.4
AB-3	47.6
AB-4	50.7
AB-5	70.0
AB-6	53.4
AB-7	60.4
aminoguanidine	8.0

Best Mode for Carrying out the Invention

Examples:

The following are examples of pharmaceutical compositions of Maillard's reaction inhibitors of the present invention. Each code in the formulae represents each of the compounds described in the section of Pharmacological test.

(Example 1) Oral tablets

According to the formula below, the ingredients are formed into a tablet by a conventional method. Sugar coating may optionally be made.

AB-1	100 mg
lactose	80 mg
corn starch	17 mg
magnesium stearate	3 mg

(Example 2) Oral tablets

According to the formula below, the ingredients are formed into a tablet by a conventional method. Sugar coating may optionally be made.

AB-2	50 mg
corn starch	90 mg
lactose	30 mg
hydroxypropylcellulose	25 mg
magnesium stearate	5 mg

(Example 3) Capsules

According to the formula below, the ingredients are admixed and granulated by a conventional method and filled in capsules in an amount of 100 mg/capsule.

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AB-3	10 mg
corn starch	45 mg
lactose	20 mg
crystalline cellulose	24 mg
talc	0.5 mg
magnesium stearate	0.5 mg

10 (Example 4) Injection

According to the formula below, the ingredients are admixed by a conventional method to dissolve. The solution is filtered, filled into vials and autoclaved to sterilize.

AB-4	20 mg
chlorobutanol	5 mg
water for injection	1 ml

20 (Example 5) Eye drops

According to the formula below, the ingredients are admixed by a conventional method to dissolve, and the solution is sterilized by filtration.

AB-5	0.5 g
boric acid	1.0 g
borax	q.s.(pH 7.0)
sodium chloride	0.25 g
disodium edetate	0.02 g
chlorobutanol	0.2 g
polysorbate 80	0.2 g
sodium sulfite	0.2 g
sterile purified water	to 100 ml

(Example 6) Eye ointment

40 According to the formula below, the ingredients are admixed by a conventional method to form an eye ointment.

AB-7	0.5 g
white vaseline	100 g

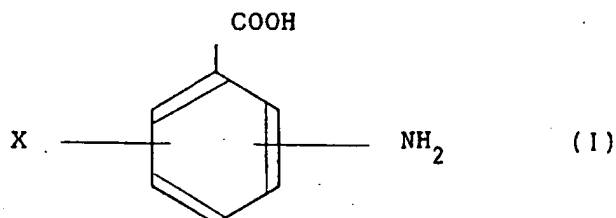
Industrial Applicability

50 The inhibitors of Maillard's reaction represented by the formula (I) and pharmaceutically acceptable salts thereof, inhibit the very formation of Amadori rearrangement product, the immediate causing factor of cross linkings among protein molecules.

The pharmaceutical compositions of the present invention, accordingly, may be useful for treatment and prophylaxis of diabetic complications, for example coronary heart disease, peripheral circulation disorders, cerebrovascular disorders, neuropathy, nephropathy, arteriosclerosis, arthrosclerosis, cataract and retinopathy, and age-associated disorders such as atherosclerosis, coronary heart disease, cerebrovascular disorders and senile cataract.

Claims

1. A Maillard's reaction inhibitor composition characterized in that it contains a compound represented by the formula (I),



wherein X denotes a hydroxyl group or a nitro group, a pharmaceutically acceptable ester thereof or a pharmaceutically acceptable salt of the said compound or the said ester.

2. The Maillard's reaction inhibitor composition of Claim 1 wherein the compound represented by the formula (I) is 5-hydroxyanthranilic acid.
3. The Maillard's reaction inhibitor composition of Claim 1 wherein the compound represented by the formula (I) is 3-hydroxyanthranilic acid.
4. The Maillard's reaction inhibitor composition of Claim 1 wherein the compound represented by the formula (I) is 4-nitroanthranilic acid.
5. The Maillard's reaction inhibitor composition of Claim 1 wherein the compound represented by the formula (I) is 5-aminosalicylic acid.
6. The Maillard's reaction inhibitor composition of Claim 1 wherein the compound represented by the formula (I) is 4-aminosalicylic acid.
7. The Maillard's reaction inhibitor composition of Claim 1 wherein the compound represented by the formula (I) is 3-aminosalicylic acid.
8. The Maillard's reaction inhibitor composition of Claim 1 wherein the compound represented by the formula (I) is 3-amino-4-hydroxybenzoic acid.

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP91/00200

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl ⁵ A61K31/195		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC	A61K31/195	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	JP, A, 56-7747 (May & Baker Ltd.), January 27, 1981 (27. 01. 81), (Family: none)	1-8
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed -</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
May 13, 1991 (13. 05. 91)	May 20, 1991 (20. 05. 91)	
International Searching Authority	Signature of Authorized Officer	
Japanese Patent Office		